

Transformation by the Bmi-1 Oncoprotein Correlates with Its Subnuclear Localization but Not Its Transcriptional Suppression Activity

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The *bmi-1* oncogene cooperates with *c-myc* in transgenic mice, resulting in accelerated lymphoma development. Altering the expression of Bmi-1 affects normal embryogenesis. The protein product of *bmi-1* is homologous to certain *Drosophila Polycomb* group proteins that regulate homeotic gene expression through alteration of chromatin structure. Chimeric LexA–Bmi-1 protein has previously been shown to repress transcription. How Bmi-1 functions in embryogenesis and whether this relates to the ability of Bmi-1 to mediate cellular transformation is unknown. We demonstrate here that Bmi-1 is able to transform rodent fibroblasts in vitro, providing a system that has allowed us to correlate its molecular properties with its ability to transform cells. We map functional domains of Bmi-1 involved in transcriptional suppression by using the GAL4 chimeric transcriptional regulator system. Deletion analysis shows that the centrally located helix-turn-helix-turn-helix-turn (HTHTHT) motif is necessary for transcriptional suppression whereas the N-terminal RING finger domain is not required. We demonstrate that nuclear localization requires KRMK (residues 230 to 233) and that the absence of nuclear entry ablates transformation. In addition, we find that the subnuclear localization of wild-type Bmi-1 to the rim of the nucleus requires the RING finger domain and correlates with its ability to transform. Our studies with Bmi-1 deletion mutants suggest that the ability of Bmi-1 to mediate cellular transformation correlates with its unique subnuclear localization but not its transcriptional suppression activity.

The *bmi-1* (B-cell-specific Moloney murine leukemia virus insertion site 1) oncogene was discovered by retroviral insertional mutagenesis when E μ -*myc* transgenic mice were infected with Moloney murine leukemia virus. These animals were noted to have a marked decrease in the latency period preceding the development of pre-B-cell lymphomas from approximately 150 to 50 days. Analysis of these tumors showed a variety of retroviral insertion sites with frequent integration (35 to 47%) near *bmi-1*, resulting in its overexpression (10, 39). E μ -*bmi-1* transgenic mice develop lymphomas, with a majority being of the T-cell lineage. Consistent with the retroviral insertional mutagenesis studies, crossbreeding of E μ -*bmi-1* and E μ -*myc* mice accelerates the onset of both B- and T-cell-lineage lymphomas (11).

The significance of *bmi-1* in lymphomagenesis is further underscored by its recurrent activation in naturally occurring feline lymphomas. LC-FeLV is a *myc*-containing strain of feline leukemia virus which has been shown to induce thymic lymphosarcoma in cats (23). Of these naturally occurring lymphosarcomas, 86% revealed preferential integration of LC-FeLV at a locus termed *flvi-2*, which was found to be feline *bmi-1*. The involvement of *bmi-1* in human cancers or lymphomas has not been established; however, its amino acid sequence shares 98% identity with murine Bmi-1 (23). Fluorescence in situ hybridization localizes the human *bmi-1* gene to 10p13 (2), a region involved in chromosomal translocations

associated with infant leukemias. Rearrangements in this region are also found in a subset of T-cell lymphomas (3, 5).

bmi-1 is 60% identical and 80% homologous to *mel-18*, a murine gene isolated from mouse melanoma cells. Immunostaining with purified antiserum against Mel-18 shows nuclear localization and expression in a variety of mouse tumors including T- and B-cell lymphomas (13, 37). *mel-18* appears to have tumor suppressor activity (15). Two *Drosophila* genes, *Posterior Sex Combs* and *Suppressor two of zeste*, are also homologous to *bmi-1*. These *Polycomb* group genes encode proteins responsible for restricting the expression of homeotic genes to specific domains along the anterior-posterior axis (17, 33). The action of the *Polycomb* group of gene products appears to be mediated via transcriptional suppression (4) and alteration of chromatin structure (19, 27). Furthermore, *bmi-1* has overlapping expression with *Polycomb* in *Xenopus* embryos, and their protein products appear to interact (28).

The function of *bmi-1* in the whole animal has been uncovered through studies of *bmi-1* knockout mice (38). Homozygous deletion of *bmi-1* reveals three major abnormalities. First, hematologic abnormalities include an involuted thymus, decreased spleen size, severe marrow hypoplasia, and an associated decrease in the peripheral blood counts. There is a pronounced relative increase in the number of immature thymocytes (CD4⁺/CD8⁺). B lymphocytes are the most severely affected, with no evidence of functional mature B cells. Second, neurologic abnormalities, including ataxic gait, seizures, and tremors, are noted. Histologic analysis reveals marked loss of neurons in the molecular layer of the cerebellum, a decrease in the number of Purkinje cells, and extensive gliosis of the major white matter tracts. Third, skeletal anomalies reveal posterior repositioning of each vertebra, termed

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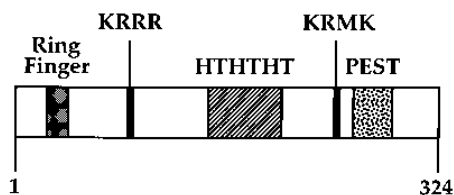


FIG. 1. Predicted structural motifs in the Bmi-1 protein. Bmi-1 is depicted by a bar representing amino acids 1 to 324. A RING finger (RING), two putative nuclear localization signals (KRRR and KRMK), and a helix-turn-helix-turn-helix-turn (HTHHTHT) motif are depicted. A region (PEST) considered to confer protein instability is shown. Adapted from references 10 and 39.

posterior transformation. Interestingly, *Eμ-bmi-1* transgenics show the reverse phenotype with anterior repositioning of each vertebra (1). These skeletal anomalies appear to reflect the mammalian counterpart of the phenomenon seen in *Drosophila melanogaster* with mutant *Polycomb* genes (1, 38).

In addition to genetic studies that have contributed significantly to an emerging picture of *bmi-1* as a gene involved in the development of body structure through gene regulation, the predicted amino acid sequence of Bmi-1 reveals several intriguing motifs. These motifs suggest that Bmi-1 functions as a transcriptional regulator. Structural motifs based on amino acid sequence analysis are depicted in Fig. 1. The N-terminal portion of the protein contains a cysteine-rich zinc finger motif with a C_3HC_4 consensus termed the RING finger (9). The RING finger has been identified in a variety of proteins including those that appear to interact with DNA, presumably as transcriptional regulatory proteins (25). In the promyelocytic leukemia gene, *pml*, the RING finger motif appears to be required for the subnuclear formation of promyelocytic leukemia oncogenic domains (8, 22). In the central portion of Bmi-1, there is a potential DNA-binding domain characterized by a helix-turn-helix-turn-helix-turn (HTHHTHT) motif (18). Bmi-1 contains two putative nuclear localization signals, KRRR and KRMK (10, 39), characterized by short stretches of basic amino acids (7). Subcellular localization by cell fractionation and immunoblotting reveals that Bmi-1 is a nuclear oncoprotein (10). The C-terminal portion of the protein contains a region with many proline, glutamic acid, serine, and threonine residues (PEST domain). Regions of this type are associated with rapid intracellular protein degradation (30).

Despite the wealth of information on *bmi-1*, the function of its protein product remains poorly understood. In this paper, we characterize functional aspects of the Bmi-1 protein. We find that Bmi-1 is sufficient by itself to transform Rat 1a cells, and we use this assay to assess the ability of specific motifs in Bmi-1 to transform cells. We demonstrate that Bmi-1 is nuclear through in situ immunofluorescence microscope studies and observe that only one of the two putative Bmi-1 nuclear localization signals is sufficient to target a cytoplasmic marker protein into the nucleus. Overexpressed Bmi-1 localizes to the outer rim of the nucleus, where heterochromatin is known to reside. Our independent studies support the findings by Bunker and Kingston (4) that Bmi-1 sequences are able to repress transcription. However, through deletion analysis, we observe a correlation between the transforming activity of Bmi-1 and its subnuclear distribution but not its ability to repress transcription.

MATERIALS AND METHODS

PCR-based cloning of *bmi-1*. Oligonucleotides (5'-AGCAGAGAAATGCATCGAAC-3' and 5'-TCCTTAACAGTCCTAAC-3') which flanked the open reading frame of the published sequence of *bmi-1* were designed. *Bam*HI and

*Eco*RI restriction sites were engineered into the oligonucleotides to facilitate subcloning of amplified DNA following PCR. The template consisted of serum-stimulated BALB/c 3T3 cDNAs (a gift of Paul Worley, Johns Hopkins University). PCRs were performed with an MJ Research thermal cycler for 30 cycles (93°C for 1 min; 50°C for 1 min; 72°C for 1 min). The resultant PCR product was subcloned into pBluescript II KS (-) (Stratagene, La Jolla, Calif.), yielding pBS/bmi, and sequenced (31) with Sequenase (U.S. Biochemical, Cleveland, Ohio) to confirm the cloning of wild-type *bmi-1*. However, sequence obtained later in the study revealed a nonconservative substitution (ATA→ATG at codon 190 of the open reading frame) generated during routine bacterial transformation. Wild-type *bmi-1* was reprepared and tested in multiple assays, confirming that the phenotype was identical to *bmi-1* containing the nonconservative substitution.

Plasmids and plasmid constructions. A eukaryotic Bmi-1 expression vector, pSG5/bmi, driven by the early simian virus 40 promoter was constructed by ligating an *Eco*RI-*Bam*HI 1.1-kb *bmi-1* fragment from pBS/bmi into the corresponding sites of pSG5 in a three-piece ligation with the pSG5 *Nde*I-*Bam*HI (1,724-bp) and pSG5 *Nde*I-*Eco*RI (2,358-bp) fragments.

pSG5/bmi deletion mutants were prepared by site-directed mutagenesis via PCR. Outside primers were the same primers engineered with *Bam*HI and *Eco*RI restriction sites used for PCR-based cloning of *bmi-1*. Internal primers for pSG5/bmi ΔRF (RING finger deletion) were 5'-CTGGACATCCATTAAAGTGGGATTAGC-3' and 5'-CACTTAATGGATGTCCAGGTTCACAAA-3'. Internal primers for pSG5/bmi ΔHT (helix-turn-helix deletion) were 5'-GTATTTCAAGCATCGTAAGTACCTTTTATC-3' and 5'-TTACGATGCTTGAAATA CAGAGTTCGGCC-3'. Internal primers for pSG5/bmi ΔNLS (nuclear localization signal deletion) were 5'-GTGACTCATGCAAGTTGGCCGAACCTG-3' and 5'-CCAAGTGCATGAGTCACAGAGGGATG-3'. Each PCR product was separately ligated in a three-piece ligation with the pSG5 *Nde*I-*Bam*HI and pSG5 *Nde*I-*Eco*RI fragments. All deletion mutants were sequenced to confirm that PCR errors were not sustained.

pGAL4/bmi was constructed by ligating a 1.1-kb *Bam*HI (Klenow blunted)-*Clal* *bmi-1* fragment from pBS/bmi into the pGALO (16) *Sal*I (Klenow-blunted) and *Clal* sites. The fusion construct was sequenced to confirm that *bmi-1* was in frame with the GAL4 sequence. pGAL4/bmi ΔRF, pGAL4/bmi ΔHT, and pGAL4/bmi ΔNLS were subcloned into pGAL4 by restriction digestion of the corresponding pSG5 mutant with *Nsi*I and *Eco*RI followed by insertion into pGAL4/bmi which had been previously digested with the same enzymes, thereby replacing full-length *bmi-1* with each deletion mutant. pG₅E1bCAT, pG₅E4CAT, and pGAL4/VP16, all kindly provided by J. Lillie and M. Green, have been described elsewhere (24). pGAL4/WT1 was kindly provided by F. Rauscher, Jr., III. pG₅TKLUC was generated by ligating the *Hind*III-*Xho*I G₅TK fragment (~150 bp) from pG₅TKCAT (a gift of F. Rauscher III) with pGL2-Basic (Promega, Madison, Wis.) *Eco*RI-*Xho*I (630-bp) and *Eco*RI-*Hind*III (4,900-bp) fragments in a three-piece ligation. pTKLUC was generated by digesting pG₅TKCAT with *Bam*HI, blunted with Klenow, and digested with *Xho*I to remove the thymidine kinase (TK) promoter-containing fragment. pGL2-Basic was digested with *Sma*I and *Xho*I and ligated with the TK fragment to yield pTKLUC.

The nuclear localization-pyruvate kinase fusion constructs were generated by ligating oligonucleotide pairs encoding putative nuclear localization signal into the *Xho*I and *Eco*RI sites of pRLPK12, a eukaryotic chicken muscle pyruvate kinase (PK) expression vector, as previously described (7). PK/KRRRa (5'-TCGAGATGAAGCGTCGACGCGATTG-3' and 5'-AATTCAAAATCGCGTCGACGCTTCATC-3') encodes amino acids EMKRRRDF; PK/KRRRb (5'-TCGAGAAAAATGAATGAAGCGTCGACGCGATG-3' and 5'-AATTCATCGCTCGACGCTTCATTCATTTTC-3') encodes amino acids KNEMKR RRD; and PK/KRMK (5'-TCGAGCCACGTCGAAAAGAATGAAGATGATG-3' and 5'-AATTCATCATCTTCATCTTTTGCACGTGGGC-3') encodes amino acids PTCKRMKMS.

Antibody preparation and protein analysis. A synthetic peptide with a cysteine tail (EDRGEVADEEKRC) encompassing amino acids 112 to 123 of murine Bmi-1 (Chiron Mimotopes, Clayton, Australia) was conjugated to keyhole limpet hemocyanin and purified by gel filtration with the Imject activated-immunogen conjugation kit (Pierce Immunotechnology, Rockland, Ill.). Antibodies were produced from two rabbits (HRP, Denver, Pa.) by using standard procedures with Freund's adjuvant. Six bleeds were obtained from each animal. Sera were tested by immunoblotting with recombinant His₆-Bmi-1 protein produced from *Escherichia coli* (4a).

Immunoblotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cellular extracts was performed as described previously (12).

Cell cultures and cell lines. Rat 1a-bmi and Rat 1a-myc/bmi cell lines were generated by lipofection (Lipofectin; Gibco BRL, Gaithersburg, Md.) of Rat 1a or Rat 1a-myc (12) cell lines with 10 μg of pSG5/bmi and 0.5 μg of pHyg, a plasmid encoding a gene conferring hygromycin resistance (36). Cells were selected in 80 μg of hygromycin per ml and passaged in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and hygromycin. Rat 1a-bmi deletion mutants were prepared in a similar fashion with pSG5/bmi ΔRF, pSG5/bmi ΔHT, or pSG5/bmi ΔNLS mutants. Stable transfectants of pG5TKLUC were obtained by lipofection of DUKXBII Chinese hamster ovary (CHO) cells with 10 μg of pG5TKLUC and 0.5 μg of pHyg. Cells were selected with hygromycin (80 μg/ml) and passaged in

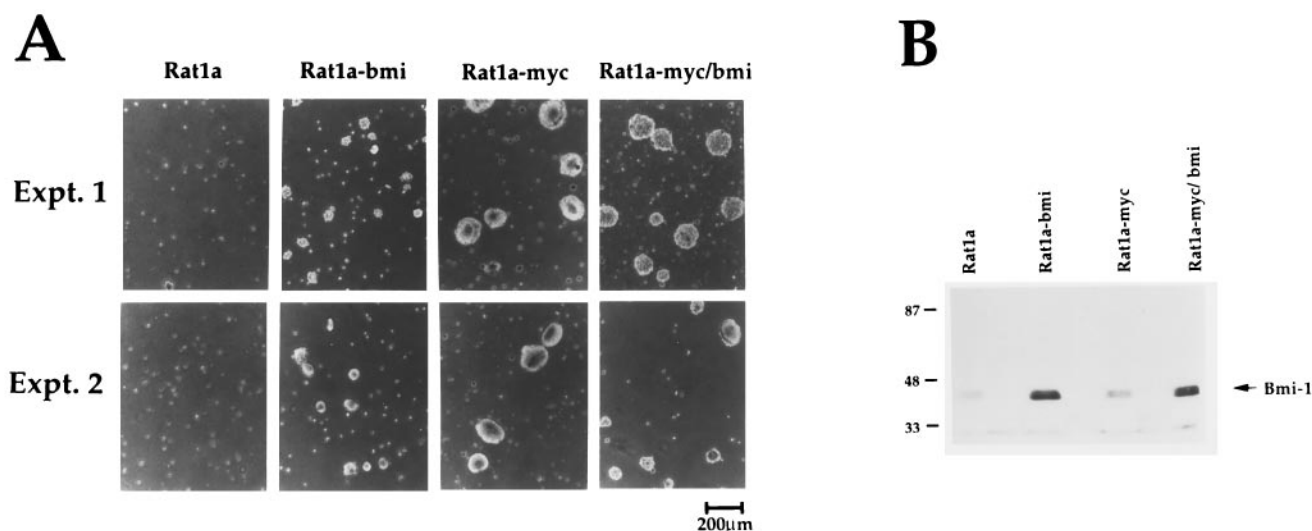


FIG. 2. Bmi-1 transforms Rat 1a fibroblasts. (A) Representative photomicrographs from two anchorage-independent growth assays of parental Rat 1a cells (hygromycin resistant in experiment 2) or Rat 1a cell lines engineered to ectopically express Bmi-1 (Rat 1a-bmi), c-Myc (Rat 1a-myc), or c-Myc and Bmi-1 (Rat 1a-myc/bmi). For experiment 2, expression of ectopic Bmi-1 alone resulted in 1,092 colonies per 100-mm dish (colony diameter, $\geq 100 \mu\text{m}$). Coexpression of Bmi-1 and c-Myc increased the cloning efficiency of the transfectants (2,232 colonies per dish) compared with expression of c-Myc alone (1,068 colonies per dish). Coexpression of both oncogenes, however, resulted in a smaller average colony size. (B) Bmi-1 is overexpressed in Rat 1a-bmi and Rat 1a-myc/bmi. An immunoblot of extracts from Rat 1a, Rat 1a-bmi, Rat 1a-myc, or Rat 1a-myc/bmi cells resolved by SDS-PAGE and probed with a rabbit polyclonal anti-Bmi-1 antibody is shown. Molecular size markers (kilodaltons) are indicated on the left.

alpha minimal essential medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and hygromycin.

Transformation assays (i) REC cotransformation assay. Rat embryo cells (REC) were harvested and grown as previously described (6, 29). For cotransformation assays, 2×10^5 REC in 75-cm² flasks were subjected to lipofection with various combinations of pMLV-c-myc (1 or 10 μg per plate), pE1ras (5 μg per plate), and pSG5/bmi (10 μg per plate) as previously described (6, 29, 35). All combinations were repeated when the amount of pMLVc-myc was reduced to 1 μg /plate. Each plasmid combination was studied in 4 to 16 separate transfected culture plates. Transformed foci were counted on days 10, 12, 14, 16, and 18 after transfection.

(ii) Anchorage independence (soft agar) assay. Rat 1a, Rat 1a-myc, Rat 1a-bmi, Rat 1a-myc/bmi, and Rat 1a-bmi deletion mutant cells (1.2×10^5) were separately plated into soft agar in 100-mm dishes as previously described (12). Experiments were repeated four to eight times.

Nuclear localization assay. The cytoplasmic marker PK expression plasmid pRLPK12 has been described previously (14). COS-7 cells were transfected with plasmids encoding PK or PK fusions by using DEAE-dextran as previously described (7). Cells on coverslips were transfected with pSG5, pSG5/bmi, pSG5/bmi Δ RF, pSG5/bmi Δ HT, or pSG5/bmi Δ NLS, fixed with paraformaldehyde, permeabilized, incubated with rabbit anti-Bmi-1 antibody, and incubated in rhodamine-goat anti-rabbit antibody. Staining of PK and immunofluorescence microscopy were performed as described previously (7).

Confocal microscopy was performed with an MRC-600 (Bio-Rad Laboratories, Hercules, Calif.) confocal imaging system. From 6 to 10 serial cross-sections were imaged from transfected cells. Images were collected and processed with CoMOS software (version 6.03; Bio-Rad).

Transcriptional assays. For all experiments, DUKXBII CHO cells were transfected with DEAE-dextran at 50% confluency as previously described (24, 29) with 2 μg of effector plasmid (pGAL4, pGAL4/BMI, pGAL4/VP16, pGAL4/WT1, or deletion mutants) and 2 μg of reporter plasmid (pG₅TKLUC, pG₅E1bCAT, pG₅E4CAT, or pTKLUC). pBluescript II KS (–) (Stratagene) was added as required to equalize the total amount of plasmid transfected. A similar strategy was employed in titration experiments. Transfection with the stable CHO/G₅TKLUC cell line was identical to that described above. The chloramphenicol acetyltransferase assay (32) and luciferase assays were performed as specified by the manufacturer (Promega, Madison, Wis.).

RESULTS

bmi-1 induces neoplastic transformation in cultured cells.

Although *bmi-1* has clearly been shown to contribute to lymphomagenesis in animals, its transforming activity in cultured cells has not been demonstrated. More importantly, the ability to assess *bmi-1* function in cultured cells facilitates studies of

its mechanism of action. To identify an assay for Bmi-1-mediated transformation, we determined the effect of *bmi-1* on Rat 1a cells, a fibroblast cell line that can be transformed by *c-myc* alone (34, 35). Rat 1a cells transformed by *c-myc* display anchorage-independent growth in soft agarose. We hypothesized that *bmi-1* would cooperate with *c-myc* in this assay and thus expected that either an increase in the number of anchorage-independent colonies or larger colonies would be observed compared with Rat 1a cells transformed by *c-myc* alone. We used Rat 1a cells, transfected Rat 1a cells overexpressing c-Myc (Rat 1a-myc), Rat 1a cells overexpressing Bmi-1 (Rat 1a-bmi), and Rat 1a cells overexpressing both c-Myc and Bmi-1 (Rat 1a-myc/bmi). The results are depicted in Fig. 2 and demonstrate two findings. First, overexpression of Bmi-1 alone (Rat 1a-bmi) is sufficient to transform cells in the absence of Myc overexpression, but the colony sizes are significantly smaller than those transformed by c-Myc. Second, the average colony size is larger in Rat 1a-myc cell lines than in Rat 1a-myc/bmi cells. However, cloning efficiency, as manifested by increased numbers of colonies per plate, is enhanced in Rat 1a-bmi cells (1,092 colonies per plate) compared with Rat 1a controls (0 colonies per plate) and in Rat1a-myc/bmi cells (2,232 colonies per plate) compared with Rat 1a-myc controls (1,068 colonies per plate). Overexpression of Bmi-1 protein in the Rat 1a-bmi and Rat 1a-myc/bmi cell lines was detected by immunoblotting (Fig. 2B). These results suggest that Bmi-1 increases the probability of anchorage independence (increase in cloning efficiency) of c-Myc-transformed Rat 1a cells. However, the expression of both oncogenes appears to inhibit the average anchorage-independent growth rate (smaller colony size). Importantly, Bmi-1 alone is able to transform Rat 1a cells, indicating that it indeed has transforming potential in the absence of c-Myc and provides a simple assay to test mutants for transforming capability.

The ability of *bmi-1* to transform REC in cooperation with *c-myc* was also assayed, since previous studies have demonstrated the ability of *c-myc* in cooperation with activated *ras* to

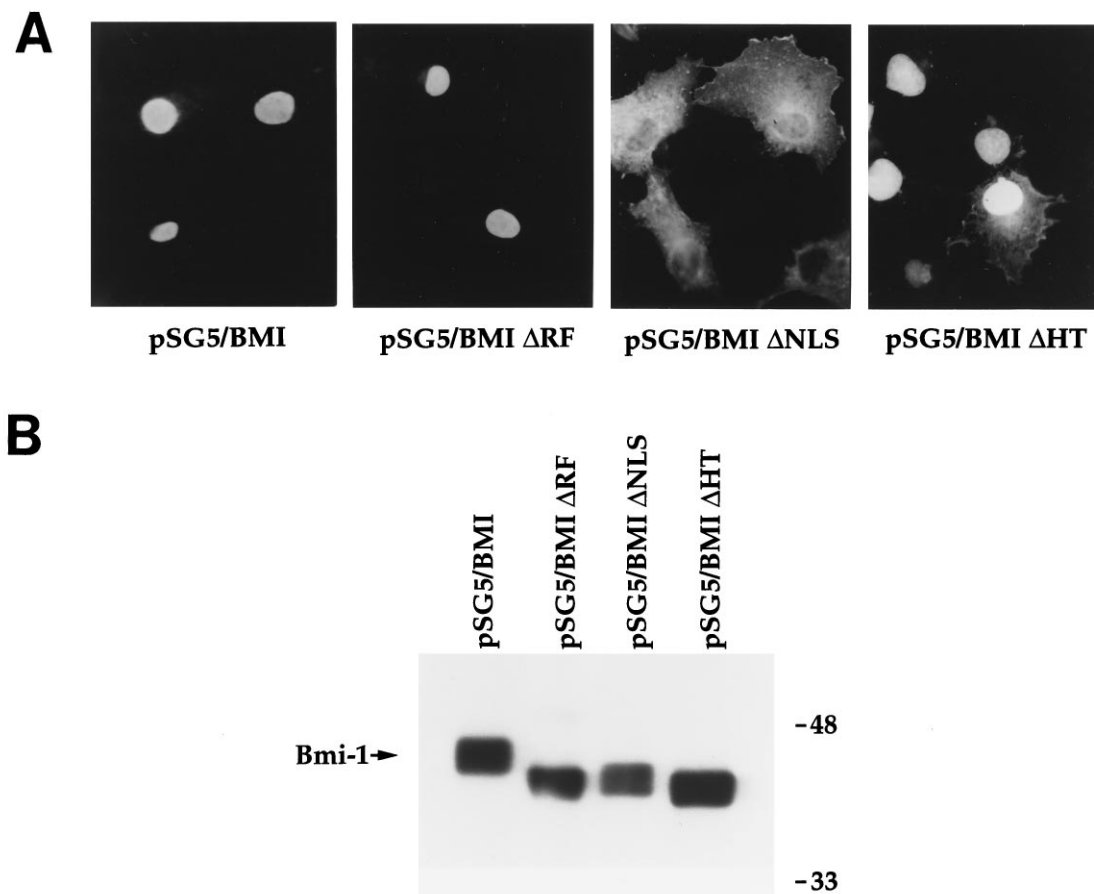


FIG. 3. Bmi-1 is localized to the nucleus of transfected COS cells. (A) COS-7 cells were transfected with the parent simian virus 40 early promoter-driven expression vector pSG5 expressing full-length Bmi-1 (pSG5/bmi) and Bmi-1 mutants with deletion of the RING finger motif (pSG5/bmi Δ RF), deletion of the nuclear localization signal (pSG5/bmi Δ NLS), or deletion of the helix-turn-helix motif (pSG5/bmi Δ HT) and stained with a polyclonal rabbit anti-Bmi-1 antibody. Immunofluorescence microscopy was performed with a rhodamine-conjugated goat anti-rabbit secondary antibody. Deletion of the nuclear localization signal results in a largely cytoplasmic distribution of Bmi-1 protein. (B) Bmi-1 and mutant proteins are expressed in COS-7 cells. An immunoblot of extracts from COS-7 cells transiently transfected with pSG5/bmi, pSG5/bmi Δ RF, pSG5/bmi Δ NLS, or pSG5/bmi Δ HT, resolved by SDS-PAGE, and probed with a rabbit polyclonal anti-Bmi-1 antibody (Fig. 2B) is shown. The positions of molecular size markers (kilodaltons) are indicated on the right.

transform primary REC (20). At standard concentrations of input plasmids, *bmi-1* did not augment the transforming activity of *myc* and *ras* (data not shown). Under the same transfection conditions, no transformed foci were noted under any of the following conditions: EJ-*ras* alone, *myc* alone, *bmi-1* alone, *ras* and *bmi-1*, or *myc* and *bmi-1* (data not shown). With a lower concentration of input *c-myc* plasmid DNA, the addition of *bmi-1* increased the number of foci (on average twofold) but did not replace the contribution of standard concentrations of *c-myc* in this assay (data not shown). These results suggest that while *bmi-1* is able to cooperate with *c-myc* in the REC co-transformation assay when a reduced concentration of *c-myc* plasmid is used, the effect of *bmi-1* in the assay is too insensitive for further transformation studies.

Nuclear localization of Bmi-1. Although previous studies based on cell fractionation suggested that Bmi-1 was a nuclear protein (10), we sought to determine its localization in situ by immunofluorescence microscopy, allowing for analysis of the subnuclear distribution by confocal microscopy. COS-7 cells were transfected with expression plasmids driven by the simian virus 40 early promoter pSG5/bmi or the parental pSG5 vector lacking an insert. Our results confirm that Bmi-1 is a nuclear protein (Fig. 3A). To be certain that the nuclear localization

was not an artifact of the high levels of protein expression generated by transient transfection in COS-7 cell lines, we further confirmed the nuclear localization of Bmi-1 by immunofluorescence with the Rat 1a-bmi stable transfectants previously described (data not shown). Attempts were made to look at the subnuclear localization of endogenous Bmi-1 in parental cell lines, but the sensitivity of the assay was insufficient for clear definition.

Nuclear localization of Bmi-1 suggests that nuclear targeting sequences are present within the protein or that Bmi-1 is tethered to another protein that bears such sequences. The predicted amino acid sequence for Bmi-1 revealed two putative nuclear localization signals (10, 39) located at residues 92 to 95 (KRRR) and 230 to 233 (KRMK). The effectiveness of each putative nuclear localization signal to function was tested by tethering each putative nuclear localization signal to the cytoplasmic marker protein chicken PK. pPK/KRMK localizes to the nucleus (data not shown). However, two other constructs encompassing different amino acids flanking KRRR are unable to localize PK to the nucleus (data not shown). To confirm that the Bmi-1 sequence containing KRMK was necessary for nuclear targeting, pSG5/bmi was mutated to delete KRMK (pSG5/bmi Δ NLS). Immunofluorescence microscopy demon-

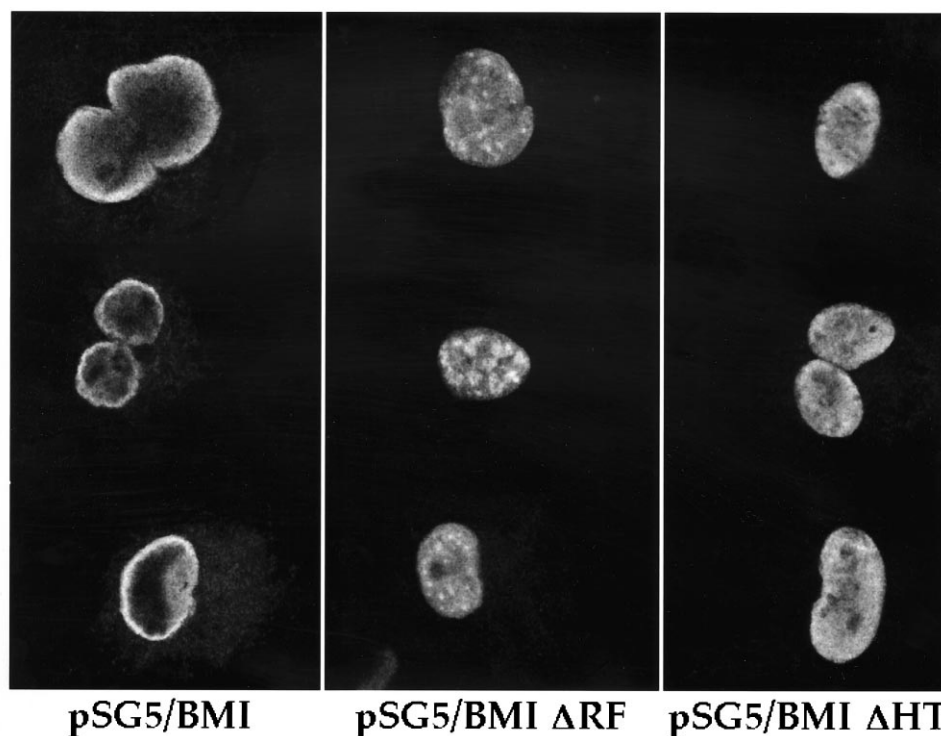


FIG. 4. Subcellular distribution of full-length Bmi-1 and deletion mutants. Bmi-1 and deletion mutants were transfected into COS-7 cells and subjected to immunofluorescence as described in the legend to Fig. 3. Confocal microscopy of representative cells reveals a prominent nuclear rim pattern for pSG5/bmi, a speckled nuclear pattern for pSG5/bmi Δ RF, and a mixed rim-homogeneous nuclear staining pattern for pSG5/bmi Δ HT. The distributions of the staining patterns are detailed in Table 1.

strates that KRMK is required for complete nuclear localization of Bmi-1 (Fig. 3A).

Similar deletion mutants were prepared by deleting the RING finger motif (pSG5/bmi Δ RF) or the helix-turn-helix motif (pSG5/bmi Δ HT). These constructs were separately transfected into COS cells to determine if these motifs affected the nuclear localization of the protein or the subnuclear localization of the protein. Neither deletion of the RING finger nor deletion of the helix-turn motif affected nuclear targeting of Bmi-1 (Fig. 3A). The transfected COS cells were examined by confocal microscopy to further characterize the subnuclear localization of each protein. Three nuclear patterns were identified: a nuclear rim pattern, a homogeneously staining pattern, and a speckled pattern. Representative cells are depicted in Fig. 4, and quantitative results are presented in Table 1. In general, pSG5/bmi-transfected COS cells adopt a nuclear rim pattern, pSG5 Δ RF-transfected cells adopt a speckled pattern, and pSG5 Δ HT-transfected cells display a mixed nuclear rim and homogeneous staining pattern.

TABLE 1. Subnuclear distribution of Bmi-1 deletion mutants

Nuclear pattern	Frequency (%) of pattern in cells with ^a :		
	BMI	Δ RF	Δ HT
Rim	84	18	52
Speckled	10	54	4
Homogeneous	6	28	44

^a Frequency of subnuclear distribution of deletion mutants as determined by confocal microscopy. Forty cells each of pSG5/bmi (BMI), pSG5/bmi Δ RF, and pSG5/bmi Δ HT were studied.

Chimeric GAL4/bmi protein suppresses transcription. During the initial phase of our studies, we were prompted by the nuclear localization of Bmi-1 to examine its potential to regulate transcription. Since LexA/Bmi-1 chimeric proteins had been shown to repress transcription (4), we focused on studies of transcriptional suppression with GAL4/Bmi-1 chimeric proteins. We sought to correlate this property of Bmi-1 with its ability to transform cells. Since a consensus DNA-binding sequence for Bmi-1 is unknown, *bmi-1* was tethered to DNA via the GAL4 DNA-binding domain in a chimeric protein comprising GAL4 and Bmi-1 sequences (pGAL4/bmi). To confirm the finding that Bmi-1 was able to suppress transcription, we constructed a reporter construct, pG₅TKLUC, containing five copies of the GAL4-binding site upstream of the TK kinase promoter driving the luciferase reporter gene. The TK promoter provides an easily detectable basal activity that allows for the detection of transcriptional suppression. CHO cells were cotransfected with pGAL4/bmi and pG₅TKLUC, and reporter activities were determined with a luciferase assay system. pGAL4/WT1, a known transcriptional suppressor (26) with the Wilms' tumor 1 cDNA tethered to GAL4, was used as a control for transcriptional suppression, and pGAL4/VP16 was used as a control for transcriptional activation. *bmi-1* sequences linked to GAL4 inhibited TK-driven transcription as efficiently as did pGAL4/WT1 (Fig. 5A). This activity was dependent on the GAL4 sites, since the control experiments with a reporter lacking GAL4 sites (pTKLUC) failed to demonstrate suppression when transfected with pGAL4/bmi (Fig. 5B). The effect of pGAL4/bmi is dose dependent. Titration with increasing amounts of pGAL4/bmi demonstrated that higher concentrations of pGAL4/bmi resulted in a greater de-

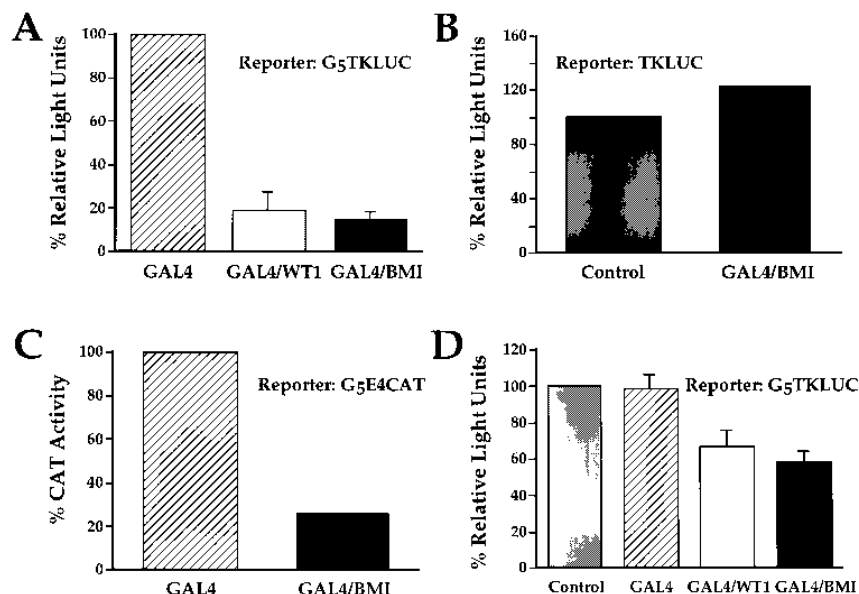


FIG. 5. Bmi-1 protein tethered to DNA represses the transcription of heterologous promoters in CHO cells. Bmi-1 sequences were fused to the yeast GAL4 DNA-binding domain and compared with GAL4 alone or GAL4 fused to the Wilms' tumor 1 (WT1) protein, a known transcriptional repressor. In all cases, effector and reporter plasmids were transiently transfected with 0.4 μ g of each plasmid per ml of transfection medium. (A) Bmi-1 fused to GAL4 (GAL4/BMI) represses pG₅TKLUC, a luciferase reporter gene driven by the TK promoter preceded by five GAL4 sites, compared with GAL4 only. GAL4/WT1 serves as a control for transcriptional suppression. The results are the means of 12 experiments, and standard errors are shown. (B) GAL4/BMI does not repress the TK promoter in the absence of GAL4 sites in the TKLUC reporter. The left bar represents the basal TKLUC activity, and the right bar shows the reporter activity in the presence of GAL4/BMI. Data are means of duplicate experiments. (C) GAL4/BMI inhibits pG₅E4CAT, a chloramphenicol acetyltransferase reporter gene driven by the adenovirus E4 promoter preceded by GAL4 sites, compared with GAL4 only. Data shown are means of duplicate experiments. (D) GAL4/BMI inhibits transcription of stably integrated pG₅TKLUC. The results (means of quadruplicate experiments with standard errors shown) represent the activities of a reporter pG₅TKLUC cell line after transient transfection with Bluescript (Control), pGAL4, pGAL4/WT1, or pGAL4/BMI.

gree of transcriptional suppression (data not shown). We determined that transcriptional suppression by pGAL4/bmi is not specific for the TK promoter since it also suppresses pG₅E4CAT (24), a reporter containing five GAL4 DNA-binding sites linked to the adenovirus E4 promoter driving the CAT gene (Fig. 5C). These results confirm that Bmi-1, when tethered to DNA, is capable of transcriptional suppression of heterologous promoters.

Since the *Drosophila* homologs *Posterior sex combs* and *Suppressor two of zeste* suppress transcription through alteration of chromatin structure, we investigated whether pGAL4/bmi was able to suppress the reporter pG₅TKLUC when integrated into the CHO cell genome. Stably transfected CHO cell clones were selected for high basal levels of luciferase activity, and pooled transfectants were used in transient transfections with pGAL4, pGAL4/bmi, or pGAL4/WT1 to determine whether *bmi-1* also represses the transcription of genomic targets. Suppression of genomic pG₅TKLUC by pGAL4/bmi was clearly detectable (Fig. 5D). Titration experiments indicated that increasing concentrations of effector plasmid produced a greater degree of transcriptional suppression (data not shown). These data suggest that pGAL4/bmi, similar to GAL4/WT1, is a transcriptional suppressor of genomic targets.

The predicted Bmi-1 amino acid sequence suggests the existence of at least two motifs, RING finger and HTHTHT, that may be involved in protein-protein or protein-nucleic acid interaction. Using the mammalian two-hybrid assay, we could not detect an interaction of Bmi-1 with itself or with c-Myc (data not shown). Since these motifs may be involved in interactions with other proteins to mediate transcriptional suppression, we prepared GAL4 fusions (Fig. 6) with isolated deletion of the RING finger (pGAL4/bmi Δ RF), the helix-turn-helix motif (pGAL4/bmi Δ HT), the KRMK nuclear targeting se-

quence (pGAL4/bmi Δ NLS), and a double mutant of the RING and helix-turn motif (pGAL4/bmi Δ RF/HT). Luciferase assays were performed with the G₅TKLUC reporter. The helix-turn motif appears necessary for suppression. Deletion of the RING finger motif does not affect the ability of the fusion construct to suppress the reporter (Fig. 6).

The RING finger, but not the helix-turn motif, is required for transformation. Having characterized the contribution of specific motifs to transcriptional suppression and subnuclear localization, we examined the role of each motif in its ability to transform Rat 1a cells. Stably transfected single-cell clones of Rat 1a transfectants were prepared by using the pSG5/bmi deletion mutants to generate cell lines with targeted deletion of the RING finger (Rat 1a-bmi Δ RF), the helix-turn-helix motif (Rat 1a-bmi Δ HT), or the KRMK nuclear targeting sequence (Rat 1a-bmi Δ NLS). Following confirmation by immunoblotting of protein expression (data not shown), at least three different single-cell clones were placed in soft agar for characterization of anchorage-independent growth as described above. Representative results, depicted in Fig. 7, demonstrate that while absence of the helix-turn motif does not affect the ability of Bmi-1 to transform cells, absence of the RING finger motif or the nuclear localization signal eliminates the ability of Bmi-1 to transform cells.

DISCUSSION

The *bmi-1* gene, which is homologous to certain *Drosophila Polycomb* group genes, has been found to contribute to transgenic animal lymphomagenesis (10, 11, 39) as well as naturally occurring feline leukemias in cooperation with *c-myc* (23). Although its contribution to human hematopoietic neoplasms remains unestablished, translocations affecting 10p13, where

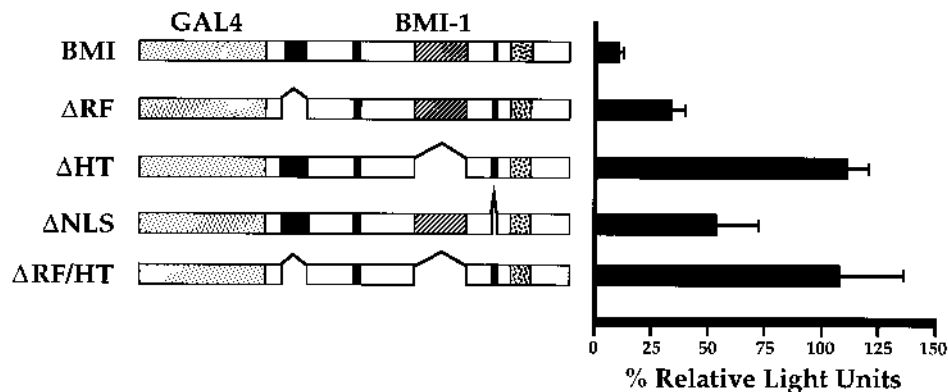


FIG. 6. The helix-turn motif, but not the RING finger motif, is required for transcriptional suppression. GAL4/bmi fusions are depicted by bars on the left side of the figure. The GAL4(1-147) DNA-binding domain (stippled) was fused to full-length Bmi-1 (top bar) or to targeted deletion of the RING finger (Δ RF), the helix-turn motif (Δ HT), the nuclear localization sequence (Δ NLS), or a combined RING finger/helix-turn motif (Δ RF/HT). The relative activities (means of three experiments with standard errors indicated) of these GAL4 hybrids with pG₅TKLUC as the reporter are indicated on the right. Results are normalized to the activity of the reporter construct transfected with pBluescript.

bmi-1 is located, have been reported in childhood acute lymphocytic leukemia (3, 5). Furthermore, the genetic interaction between *Drosophila Polycomb* and *Trithorax*, which has a region of homology with the human *MLL* leukemia gene, to regulate homeotic gene expression suggests an intriguing possible connection between *bmi-1* and *MLL* in the genesis of hematopoietic neoplasms (40). Given the significance of *bmi-1* in the development of hematopoietic neoplasms, we have undertaken studies to identify the functional domains of Bmi-1.

Bmi-1 transforming activity has to date been demonstrated experimentally only in lymphocytes of transgenic animals. Since the oncogenic activity of Bmi-1 has been shown only in vivo, we first sought to establish in vitro tissue culture assays for Bmi-1-transforming activity in order to correlate Bmi-1 structural domains with its function. We show that Bmi-1 is able to transform Rat 1a cells in the absence of ectopic c-Myc expression. In the presence of overexpressed c-Myc, Bmi-1 appears to increase the clonogenicity of these cells in soft agarose; however, the colonies were smaller than those expressing c-Myc alone. Since Rat 1a is a cell line, albeit untransformed, Bmi-1 is speculated to cooperate with some genetic defect sustained in Rat 1a cells that also allows transformation by c-Myc alone. Although the nature of the inherent defect of

Rat 1a cells is unknown, this cell line provides a convenient assay to assess the ability of Bmi-1 and its mutants to transform cells. Using this assay, we find that the RING finger domain is required for transformation of Rat 1a cells but that the HTH-THT domain is not required to transform Rat 1a cells.

To begin to understand the molecular properties of Bmi-1, we studied several aspects of Bmi-1 function as predicted from its amino acid sequence. Analysis of this sequence reveals an N-terminal RING finger domain, a central helix-turn-helix repeat, and two potential nuclear localization signals. The contribution of these domains to Bmi-1 function has been unknown. While two putative nuclear localization signal motifs have been previously recognized in Bmi-1 (10, 39), we found that only one of them (PTCKRMKMS) is capable of targeting the chimeric chicken PK protein to the nucleus. Deletion of this sequence from full-length Bmi-1 results in a cytoplasmic distribution of the mutant protein, indicating that the sequence containing KRMK is necessary for nuclear localization.

We used confocal immunofluorescence microscopy to define the subnuclear compartmentation of Bmi-1, since the subnuclear locations of proteins such as the promyelocytic leukemia protein are critical for their function (8, 22). While we would have preferred to use endogenous Bmi-1 or ectopically

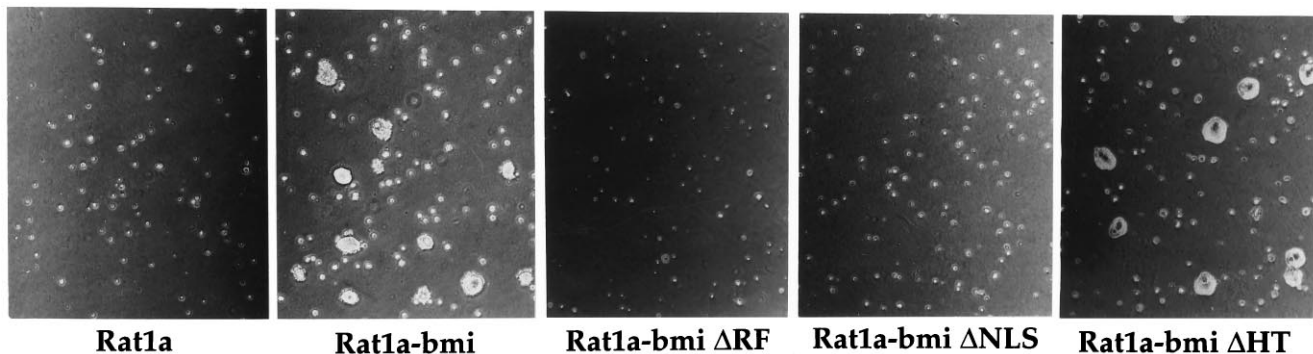


FIG. 7. The RING and nuclear localization sequence, but not the helix-turn motif, are required to transform Rat 1a fibroblasts. The five panels depict representative photomicrographs from three anchorage-independent growth assays of parental Rat 1a cells, Rat 1a cell lines engineered to ectopically express full-length Bmi-1 (Rat 1a-bmi), Bmi-1 with deletion of the RING finger motif (Rat 1a-bmi Δ RF), Bmi-1 with deletion of the nuclear localization sequence (Rat 1a-bmi Δ NLS), or Bmi-1 with deletion of the helix-turn motif (Rat 1a-bmi Δ HT). As seen in Fig. 2, Rat 1a-bmi cells are able to form colonies in soft agar. Deletion of the RING finger or nuclear localization sequence ablated the ability of Bmi-1 to enable these cells to grow in soft agar. Single-cell clones of Rat 1a-bmi Δ HT were able to form colonies in soft agar.

expressed Bmi-1 in the Rat 1a cell lines for subnuclear localization studies, Bmi-1 expression in these cells was insufficient for high-resolution confocal immunofluorescence microscopy. Therefore, we overexpressed Bmi-1 and all deletion mutants in COS-7 cells. Since protein expression is comparable for all constructs (Fig. 3B), variations in subnuclear localization presumably depend on specific properties of Bmi-1 and the deletion mutants, as opposed to being an artifact due to overexpression of the protein product. Wild-type Bmi-1 localizes to the nuclear rim, where heterochromatin is known to reside. Deletion of the HTHTHT motif increases the diffuseness of this mutant; however, it remains predominantly at the nuclear rim. In contrast, the vast majority of cells expressing the Bmi-1 deletion mutant lacking the RING finger display a speckled distribution of the mutant Bmi-1 protein. We conclude from these high-resolution localization experiments that specific domains of Bmi-1 appear to influence the subnuclear localization of Bmi-1 and that the RING finger domain appears to be required for the nuclear rim distribution noted with the full-length protein.

A number of possible functions for Bmi-1 may be inferred from its location in the nucleus. Since Bunker and Kingston (4) have demonstrated the ability of these proteins to repress transcription in transient-transfection experiments, we sought to correlate this activity with the ability of Bmi-1 to transform cells. The transcriptional properties of Bmi-1 were studied in the context of chimeric GAL4 proteins, since the target DNA site for Bmi-1, if any exists, is unknown. We found that Bmi-1 is able to inhibit transcription in transient transfections. In addition to confirming earlier studies (4), we observed that GAL4/Bmi-1 is a repressor of the chromosomally integrated reporter. Our results indicate that the ability of GAL4/Bmi-1 to repress transcription requires the HTHTHT domain but not the RING finger motif.

Our analysis of the functional domains of Bmi-1 suggests that the sequence KRMK targets Bmi-1 into the nucleus. Once translocated into the nucleus, the RING finger domain is required for localization of Bmi-1 to the rim of the nucleus. The HTHTHT domain is required for the ability of GAL4/Bmi-1 to repress transcription in a GAL4-dependent transient-transfection system. These studies allow us to correlate functional domains of Bmi-1 with its ability to induce anchorage-independent growth of Rat 1a cells. Removal of the nuclear targeting sequence KRMK abrogates the ability of Bmi-1 to transform, indicating that nuclear localization is necessary for transformation. Furthermore, the rim pattern of subnuclear location of Bmi-1, which requires the RING finger domain, correlates with its transforming activity. Intriguingly, the transcriptional suppression activity of Bmi-1, which is dependent on the HTHTHT motif, does not correlate with its ability to transform rodent fibroblasts. This observation is interesting specifically since the only known common activity between Bmi-1 and its *Drosophila* homologs is transcriptional suppression in transient-transfection experiments. Our results suggest the possibility that overexpression of Bmi-1 contributes to neoplastic transformation through mechanisms not directly related to its normal function in development.

The homology of Bmi-1 with the *Drosophila* Polycomb group proteins suggests that it may play a role in regulating homeotic gene expression and thereby altering skeletal organization in transgenic *bmi-1* animals. Since alterations of *HOX* genes by way of chromosomal translocations are found in leukemias (21), it is possible that the contribution of Bmi-1 to lymphomagenesis involves alteration of *HOX* gene expression. Intriguingly, the leukemia gene *MLL*, which has homology to *Trithorax*, is also involved in homeotic gene expression, and its

absence also results in skeletal abnormalities (40). In *D. melanogaster*, however, the Polycomb group of proteins inhibits homeotic gene expression whereas *Trithorax* activates homeotic gene expression (17). Thus, it is possible that Bmi-1 cooperates with c-Myc by activating pathways that coincide with that of the leukemia-related gene *MLL*. Our studies have correlated the transforming activity of Bmi-1 with nuclear localization to the rim but not with its ability to repress transcription. These observations provide the foundation to further investigate the molecular mechanisms underlying the ability of Bmi-1 to mediate neoplastic transformation.

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REFERENCES

1. Alkema, M. J., N. M. T. van der Lugt, R. C. Bobeldijk, A. Berns, and M. van Lohuizen. 1995. Transformation of axial skeleton due to overexpression of *bmi-1* in transgenic mice. *Nature* (London) **374**:724–727.
2. Alkema, M. J., J. Wiegant, A. K. Raap, A. Berns, and M. van Lohuizen. 1993. Characterization and chromosomal localization of the human proto-oncogene *BMI-1*. *Hum. Mol. Genet.* **2**:1597–1603.
3. Berger, R., L. Baranger, A. Bernheim, F. Valensi, and G. Flandrin. 1987. Cytogenetics of T-cell malignant lymphoma. Report of 17 cases and review of the chromosomal breakpoints. *Cancer Genet. Cytogenet.* **36**:123–130.
4. Bunker, C. A., and R. E. Kingston. 1994. Transcriptional repression by *drosophila* and mammalian polycomb group proteins in transfected mammalian cells. *Mol. Cell. Biol.* **14**:1721–1732.
- 4a. Cohen, K. J., and C. V. Dang. Unpublished data.
5. D'Alessandro, E., P. Paterlini, M. Lo Re, M. Di Cola, C. Ligas, D. Quagliano, and G. Del Porto. 1990. Cytogenetic follow-up in a case of Sezary syndrome. *Cancer Genet. Cytogenet.* **45**:231–236.
6. Dang, C. V., J. Barrett, M. Villa-Garcia, L. M. S. Resar, G. J. Kato, and E. R. Fearon. 1991. Intracellular leucine zipper interactions suggest c-Myc heterooligomerization. *Mol. Cell. Biol.* **11**:954–962.
7. Dang, C. V., and W. M. F. Lee. 1989. Nuclear and nucleolar targeting sequences of c-erb-A, c-myc, p53, HSP70 and HIV tat proteins. *J. Biol. Chem.* **264**:18019–18023.
8. Dyck, J. A., G. G. Maul, W. H. Miller, Jr., J. D. Chen, A. Kakizuka, and R. M. Evans. 1994. A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell* **76**:333–343.
9. Freemont, P. S., I. M. Hanson, and J. Trowsdale. 1991. A novel cysteine-rich sequence motif. *Cell* **64**:483–484.
10. Haupt, Y., W. S. Alexander, G. Barri, S. P. Klinken, and J. M. Adams. 1991. Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in Eμ-myc transgenic mice. *Cell* **65**:753–763.
11. Haupt, Y., M. L. Bath, A. W. Harris, and J. M. Adams. 1993. *bmi-1* transgene induces lymphomas and collaborates with myc in tumorigenesis. *Oncogene* **8**:3161–3164.
12. Hoang, A. T., K. J. Cohen, J. F. Barrett, D. A. Bergstrom, and C. V. Dang. 1994. Participation of cyclin A in myc-induced apoptosis. *Proc. Natl. Acad. Sci. USA* **91**:6875–6879.
13. Ishida, A., H. Asano, M. Hasegawa, H. Koseki, T. Ono, M. C. Yoshida, M. Taniguchi, and M. Kanno. 1993. Cloning and chromosome mapping of the human *Mel-18* gene which encodes a DNA-binding protein with a new 'RING-finger' motif. *Gene* **129**:249–255.
14. Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell* **39**:499–509.
15. Kanno, M., M. Hasegawa, A. Ishida, K. Isono, and M. Taniguchi. 1995. *mel-18*, a Polycomb group-related mammalian gene, encodes a transcriptional negative regulator with tumor suppressive activity. *EMBO J.* **14**:5672–5678.
16. Kato, G. J., J. Barrett, M. Villa-Garcia, and C. V. Dang. 1990. An amino-terminal c-myc domain required for neoplastic transformation activates transcription. *Mol. Cell. Biol.* **10**:5914–5920.
17. Kennison, J. A. 1995. The polycomb and trithorax group proteins of *Drosophila*: trans-regulators of homeotic gene function. *Annu. Rev. Genet.* **29**:289–303.

18. **Klemsz, M. J., S. R. McKercher, A. Celada, C. Van Beveren, and R. A. Maki.** 1990. The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell* **61**:113–124.
19. **Krumlauf, R.** 1994. Hox genes in vertebrate development. *Cell* **78**:191–201.
20. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (London)* **304**:596–602.
21. **Lawrence, H. J., and C. Largman.** 1992. Homeobox genes in normal hematopoiesis and leukemia. *Blood* **80**:2445–2453.
22. **Le, X. F., P. Yang, and K. S. Chang.** 1996. Analysis of the growth and transformation suppressor domains of promyelocytic leukemia gene, pml. *J. Biol. Chem.* **271**:130–135.
23. **Levy, L. S., P. A. Lobelle-Rich, and J. Overbaugh.** 1993. flvi-2, a target of retroviral insertional mutagenesis in feline thymic lymphosarcomas, encodes bmi-1. *Oncogene* **8**:1833–1838.
24. **Lillie, J. W., and M. R. Green.** 1989. Transcription activation by the adenovirus E1A protein. *Nature (London)* **338**:39–44.
25. **Lovering, R., I. M. Hanson, K. L. B. Borden, S. Martin, N. J. O'Reilly, G. I. Evan, D. Rahman, D. J. C. Pappin, J. Trowsdale, and P. S. Freemont.** 1993. Identification and preliminary characterization of a protein motif related to the zinc finger. *Proc. Natl. Acad. Sci. USA* **90**:2112–2116.
26. **Madden, S. L., D. M. Cook, and F. J. Rauscher.** 1993. A structure-function analysis of transcriptional repression mediated by the WT1, Wilms' tumor suppressor protein. *Oncogene* **8**:1713–1720.
27. **Müller, J.** 1995. Transcriptional silencing by the Polycomb protein in *Drosophila* embryos. *EMBO J.* **14**:1209–1220.
28. **Reijnen, M. J., K. M. Hamer, J. L. den Blaauwen, C. Lambrechts, I. Schoneveld, R. van Driel, and A. P. Otte.** 1995. Polycomb and bmi-1 homologs are expressed in overlapping patterns in *Xenopus* embryos and are able to interact with each other. *Mech. Dev.* **53**:35–46.
29. **Resar, L. M. S., C. Dolde, J. F. Barrett, and C. V. Dang.** 1993. B-Myc inhibits neoplastic transformation and transcriptional activation by c-Myc. *Mol. Cell. Biol.* **13**:1130–1136.
30. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**:364–368.
31. **Sanger, F., S. Nicklen, and A. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
32. **Seed, B., and J. Y. Sheen.** 1988. A simple phase-extraction assay for chloramphenicol acetyltransferase activity. *Gene* **67**:271.
33. **Simon, J., A. Chiang, W. Bender, M. J. Shimell, and M. O'Connor.** 1993. Elements of the *Drosophila* bithorax complex that mediate repression by Polycomb group products. *Dev. Biol.* **158**:131–144.
34. **Small, M. B., N. Hay, M. Schwab, and J. M. Bishop.** 1987. Neoplastic transformation by the human gene N-myc. *Mol. Cell. Biol.* **7**:1638–1645.
35. **Stone, J., T. de Lange, G. Ramsay, E. Jakobovits, J. M. Bishop, H. E. Varmus, and W. M. F. Lee.** 1987. Definition of regions in human c-myc that are involved in transformation and nuclear localization. *Mol. Cell. Biol.* **7**:1697–1709.
36. **Sugden, B., K. Marsh, and J. Yates.** 1985. A vector that replicated as a plasmid can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. *Mol. Cell. Biol.* **5**:410–413.
37. **Tagawa, M., T. Sakamoto, K. Shigemoto, H. Matsubara, Y. Tamura, T. Ito, I. Nakamura, A. Okitsu, K. Imai, and M. Taniguchi.** 1990. Expression of novel DNA-binding protein with zinc finger structure in various tumor cells. *J. Biol. Chem.* **265**:20021–20026.
38. **van der Lugt, N. M. T., J. Domsen, K. Linders, M. van Roon, E. Robanus-Maandag, H. te Riele, M. van der Valk, J. Deschamps, M. Sofroniew, M. van Lohuizen, and A. Berns.** 1994. Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. *Genes Dev.* **8**:757–769.
39. **van Lohuizen, M., S. Verbeek, B. Scheijen, E. Wientjens, H. van der Gulden, and A. Berns.** 1991. Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging. *Cell* **65**:737–752.
40. **Yu, B. D., J. L. Hess, S. E. Horning, G. A. J. Brown, and S. J. Korsmeyer.** 1995. Altered Hox expression and segmental identity in MLL-mutant mice. *Nature (London)* **378**:505–508.